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Enzymatic characteristics of ligninperoxidases from *Penicillium citrinum, Fusarium oxysporum* and *Aspergillus terreus* using *n*-propanol as substrate

Meera Yadav and K D S Yadav*

Department of Chemistry, D D U Gorakhpur University, Gorakhpur 273 009, India

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The activities of ligninperoxidases from *Penicillium citrinum* MTCC 3565*, Fusarium oxysporum* MTCC 3379 and *Aspergillus terreus* MTCC 3374 have been assayed and the enzymatic characteristics like K_m, *p*H and temperature optima using *n*-propanol as the substrate have been reported. The results suggest that *n*-propanol can substitute veratryl alcohol as substrate for assaying ligninperoxidase activities from different fungal strains, without affecting the enzymatic characteristics. The above strains were selected, as they were known to secrete ligninperoxidase in the liquid culture medium.

Keywords: Ligninperoxidases, enzymatic characteristics, *n*-propanol, veratryl alcohol, *Penicillium citrinum*, *Fusarium oxysporum*, *Aspergillus terreus*

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Ligninperoxidases [E.C.1.11.1.7] catalyze the H_2O_2-dependent oxidation of a variety of lignin model compounds[1]. They find potential applications in delignification of lignocellulosic materials[2], in conversion of coal to low molecular mass fractions[3] which could be used as feed-stock for the production of commodity chemicals, in bio-pulping and bio-bleaching[4] in paper industries, in removal of recalcitrant organic pollutants[5-7] and in enzymatic polymerisation[8] in polymer industries. A number of methods for assaying the activity of ligninperoxidases have been developed[9] which use monomeric and dimeric lignin model compounds, polymeric dyes and [^{14}C] and [^{13}C]-labelled lignins. However, these methods either involve rare or expensive substrates like [^{14}C] and [^{13}C]-labelled lignins or lack wide applicability. The most convenient among the available assay methods is based on using veratryl alcohol as substrate and monitoring the formation of corresponding aldehydes spectrophotometrically at 310 nm. However, even this substrate is not available in pure form commercially and the commercially available form needs vacuum distillation before use, thus is not a convenient substrate for assaying the activity of ligninperoxidase. Therefore, there is a need to develop a convenient substrate for assaying the activity of ligninperoxidase.

Earlier, we demonstrated that *n*-propanol could act as a better substrate than veratryl alcohol for assaying ligninperoxidase from *Phanerochaete chrysosporium*[10]. In continuation of the earlier study, in the present paper, *n*-propanol is used as the substrate for assaying the activities of ligninperoxidases from other fungal strains namely *Penicillium citrinum* MTCC 3565*, Fusarium oxysporum* MTCC 3379 and *Aspergillus terreus* MTCC 3374. Also, the enzymatic characteristics like K_m, *p*H and temperature optima of ligninperoxidases using *n*-propanol as the substrate from these strains have been reported, in order to identify the suitable enzymes for biotechnological applications.

Materials and Methods

Veratryl alcohol (3,4-dimethoxy benzyl alcohol) was obtained from Aldrich (Wisconsin, USA). Dimethyl succinate and nitrilotriacetate were from Sigma Chemical Co., St. Louis USA. All other chemicals were obtained from CDH (Delhi) or Loba Chemie (Mumbai) and used without further purification.

Fungal strains

Penicillium citrinum MTCC 3565*, Fusarium oxysporum* MTCC 3379 and *Aspergillus terreus* MTCC 3374 were isolated using pour plate technique[11] from soil samples, collected from sites, where waste water of Sanjai Paper and Chemical Industries Khalilabad, Basti (U. P.), India was being discharged. The medium used[9] consisted of glucose (10 g), malt extract (10 g), peptone (2 g), yeast extract (2 g), L-asparagine (1 g), KH_2PO_4 (2 g), $MgSO_4.7H_2O$ (1 g), thiamine-HCl (1 mg) and agar (20 g) dissolved in double distilled water (1 L).

The purified microorganisms were tested for extra-cellular secretion of ligninperoxidase in liquid culture growth medium. The growth medium consisted of 10

*Corresponding author
Phone: (0551) 202349 (R)
Fax: (0551) 340459
E mail: kds_chemistry@rediffmail.com

g glucose, 1.32 g ammonium tartarate, 0.2 g KH_2PO_4, 50 mg $MgSO_4.7H_2O$, 10 mg $CaCl_2$, 10 µg thiamine per litre and 1 ml of a solution containing per liter 3 g $MgSO_4.7H_2O$, 0.5 g $MnSO_4.H_2O$, 1 g NaCl, 100 mg $FeSO_4.7H_2O$, 185 mg $CoCl_2.6H_2O$, 80 mg $CaCl_2$, 180 mg $ZnSO_4.7H_2O$, 10 mg $CuSO_4.5H_2O$, 10 mg $AlK(SO_4)_2$, 10 mg H_3BO_3, 12 mg $Na_2MoO_4.2H_2O$ and 1.5 g nitrilotriacetate. The *p*H of basal medium was adjusted to 4.5 with 20 m*M* dimethyl succinate. Growth media containing natural lignin sources like coirdust, sawdust, corncob, bagasse particles and wheat straw were separately prepared by adding 0.5 g of one of the natural lignin sources to 20 ml of growth medium in 100 ml culture flasks which were sterilized. The sterilized growth medium was inoculated with 1 ml of spore suspension (spore density 5×10^6 spores/ml) under aseptic condition and the fungal culture was grown under stationary culture condition at 30ºC in a incubator.

Assay of ligninperoxidase activity

The ligninperoxidase activity was assayed using veratryl alcohol as the substrate and monitoring the formation of veratraldehyde spectrophotometrically at λ=310 nm with UV/vis spectrophotometer (Hitachi, Japan, Model U-2000), fitted with electronic temperature control unit[9]. Aliquots (1 ml) of the growth medium were withdrawn at the regular intervals of 1 day and were filtered through Millipore Millex-GS 0.22 µM filter unit. Filtered growth medium (0.2 ml) was added to 0.8 ml of the reaction mixture containing veratryl alcohol (2.0 m*M*), H_2O_2 (0.40 m*M*) in 50 m*M* of tartaric acid/disodium tartrate buffer (*p*H 2.5) contained in 1 ml cuvette maintained at 25ºC.

One unit of ligninperoxidase was defined as the amount of enzyme, which converted 1 µmole of veratryl alcohol to veratraldehyde under the condition specified above. The enzyme unit was calculated using molar extinction coefficient value of 9300 M^{-1} cm^{-1} for veratraldehyde. The least count of the absorbance measurement was 0.001 absorbance unit. The values of the steady-state velocities were average of triplicate measurements with standard deviation values of 7.2%.

Preparation of ligninperoxidase

Ligninperoxidases were prepared by growing the fungal cultures (10 × 20 ml volumes) in 10 culture flasks (100 ml) as mentioned above. The maximum activity appeared on 5th day after inoculation of fungal spores. On 5th day, cultures in all the 10 flasks were pooled, filtered through four layers of cheese cloth and concentrated using Amicon concentration cell unit model 8200 and PM-10 ultrafiltration membranes (10 kD cut off value). The 10-times concentrated enzyme samples were stored at 4ºC. The enzymes did not loose appreciable activities for 1 month under these conditions.

Enzymatic characteristics of ligninperoxidase

The K_m, *p*H and temperature optima were determined using *n*-propanol as the substrate and monitoring the formation of propionaldehyde at wavelength 300 nm spectrophotometrically. The value of molar extinction coefficient of propionaldehyde[12] used for the calculation of the enzyme units was 20 $M^{-1}cm^{-1}$. For determination of K_m, steady-state velocities of the enzyme-catalyzed reaction were determined at different concentrations of *n*-propanol and K_m was calculated from the double reciprocal plot of 1/[V] vs 1/[S], where [V] is the steady-state velocity of the enzyme-catalyzed reaction and [S] is the concentration of *n*-propanol.

For determination of *p*H optimum, the steady-state velocity of the enzyme-catalyzed reaction was determined at different *p*H values and a plot of V vs *p*H was drawn. The *p*H range from 1.5 to 4.5 was maintained using tartaric acid/disodium tartrate buffer (50 m*M*). For determination of temperature optimum, steady-state velocity of the enzyme-catalyzed reaction was determined at different temperatures and a plot of V vs temp was drawn.

Results and Discussion

Like veratryl alcohol, *n*-propanol could also act as a substrate for assaying the activity of ligninperoxidase and the formation of propanaldehyde could be monitored at λ= 300 nm[12] using molar extinction value of 20 M^{-1} cm^{-1}. In this paper, *n*-propanol was used as the substrate for assaying the activity of ligninperoxidases from the fungal strains *Penicillium citrinum, Fusarium oxysporum* and *Aspergillus terreus*.

Fig. 1 shows the time-course of the variation of absorbance in reaction mixture containing ligninperoxidases from different fungal strains. As the curves platue relatively quickly (Fig. 1), only initial linear portions of the curves were relevant for the calculation of steady-state velocity. It is obvious from Fig. 1 that absorbance change per unit time is greater, where *n*-propanol is used as the substrate, as

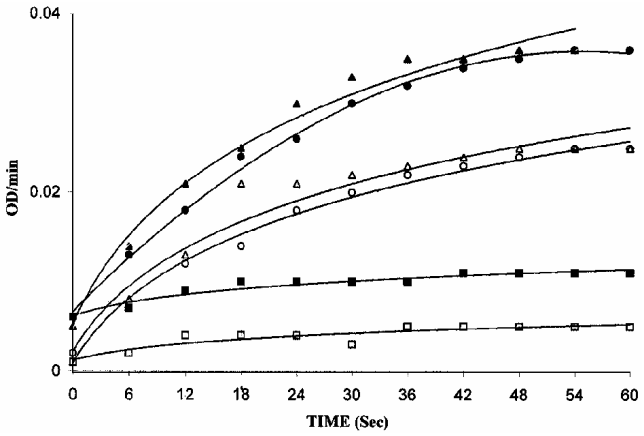

Fig. 1—Variation of absorbance with time in reaction mixtures containing ligninperoxidases from the fungal strains *Penicillium citrinum, Fusarium oxysporum* and *Aspergillus terreus* [*P. citrinum* (o) , *F. oxysporum* (Δ) and *A. terreus* (□) represent the reaction mixtures containing veratryl alcohol as the substrate where formation of veratraldehyde was monitored at λ=310 nm. *P. citrinum* (●) , *F. oxysporum* (▲) and *A. terreus* (■) represent the reaction mixtures containing *n*-propanol as the substrate, where formation of propionaldehyde was monitored at λ=300 nm]

Table 1—K_m values for ligninperoxidases of different fungal strains using *n*-propanol and veratryl alcohol as substrate

Fungal strains	K_m for *n*-propanol	K_m for veratryl alcohol
Penicillium citrinum	525 μ*M*	69 μ*M*[13]
Fusarium oxysporum	550 μ*M*	64 μ*M*[13]
Aspergillus terreus	533 μ*M*	60 μ*M*[13]
Phanerochaete chrysosporium	500 μ*M*	60 μ*M*[10]

compared to veratryl alcohol. Though the molar extinction coefficient of veratryldehyde (9300 M^{-1} cm^{-1}) is much higher than that of propanaldehyde (20 M^{-1} cm^{-1}), the rate of enzymatic reaction is much faster in case of *n*-propanol, resulting in the greater change of absorbance per unit time than using veratryl alcohol. Earlier, we demonstrated that ethanol, possibly due to its smaller size was not suitable as a substrate for assaying the activity of ligninperoxidases, whereas, the absorbance change per unit time, in case of *n*-butanol as a substrate was half of that observed in case of *n*-propanol[10]. Thus, it appears that the size of *n*-propanol fits better at the active site of ligninperoxidases and hence it is a better substrate.

The K_m values of ligninperoxidases from the different fungal strains using *n*-propanol and veratryl alcohol as the substrate are given in Table 1. It is obvious from Table 1 that K_m values using *n*-propanol are higher than that using veratryl alcohol. Since K_m value of an enzyme reflects its affinity for the

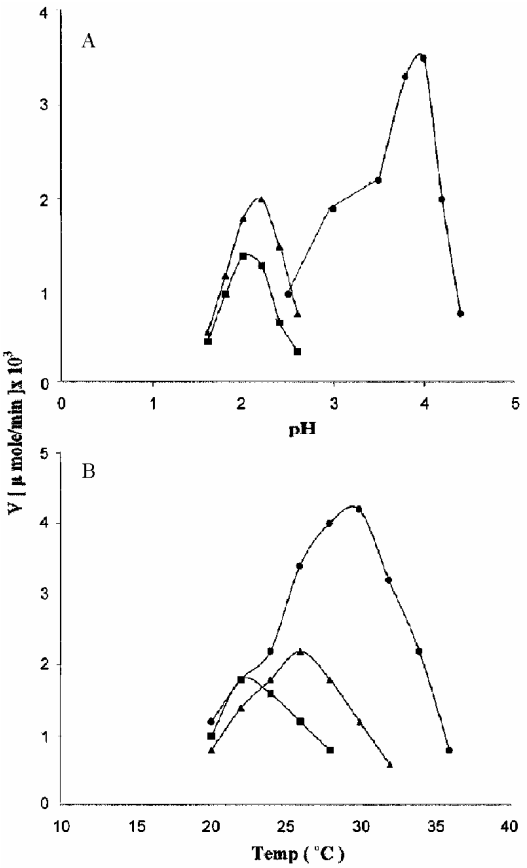

Fig. 2—Variation of the activities of the ligninperoxidases of different fungal strains with variation of reaction *p*H (A); and variation of reaction temperature (B) [*Penicillium citrinum* (●); *Fusarium oxysporum* (▲); and *Aspergillus terreus* (■)]

substrate, ligninperoxidase has lower affinity for *n*-propanol as compared to veratryl alcohol. The measurement of V_{max} indicates that the enzymatic reaction is approximately 600-times faster in case of *n*-propanol than using veratryl alcohol. As the activity of ligninperoxidases is assayed spectrophotometrically, *n*-propanol gives higher absorbance change per min than veratryl alcohol. In this way, *n*-propanol is a better substrate for assaying ligninperoxidase activity.

The variation of activity of ligninperoxidases of different fungal strains with the variation of *p*H and temperature of the reaction medium is shown in Figs 2A and B, respectively. The values for *p*H optima for ligninperoxidases of *P. citrinum, F. oxysporum* and *A. terreus* were 4.0, 2.3 and 2.0, whereas the temperature optima using *n*-propanol as the substrate were 30, 25 and 22°C, respectively. The values of *p*H and temperature optima of ligninperoxidase of the above fungal strains were similar to those reported using veratryl alcohol as the substrate[13]. However, the

wide variation in the *p*H (2.0 to 4.0) and the temperature optima (22 to 30°C) of the ligninperoxidases obtained in the present study could not be explained, thus detailed structural and functional studies are needed.

Ligninperoxidases having *p*H nearly neutral or alkaline and temperature optima around 25°C may be more suitable for delignification of lignocellulosic materials in paper industries, where paper pulp has alkaline *p*H. However, none[14] of the ligninperoxidases reported so far has *p*H optima near neutral or alkaline region. The ligninperoxidase of *P. citrinum,* having *p*H and temperature optima around 4 and 30°C, respectively may be considered relatively better suited for industrial applications.

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